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14. ABSTRACT
Our goals for this project are to explore LSD1 inhibition of protein-protein interactions as a potential therapy for $ERα^-$ breast cancer, ameliorating iLCC for *in vivo* use, and using novel proteomics approaches to identify coregulatory proteins interacting with ERα and LSD1 in $ERα^+$ cells and deduce how the complement of LSD1 associating proteins change in $ERα^-$ cancers. We have made significant progress on the aims this project, specifically by completing xenograft studies of iLCC expression and its effect on breast cancer tumor growth and on histone demethylase inhibition. We have made progress on synthesis of selective nonpeptidic LSD1 inhibitors and have initiated studies to identify optimal candidate sequences for stapled peptide analogues of iLCC. Lastly, we have conducted a detailed analysis of LSD1 fragmentation by ESI-MS and have used H/D exchange to identify the binding interface between LSD1 and a partner protein in preparation for interrogating communication between this protein and the ER. At this time there are no changes proposed for the Statement of Work.

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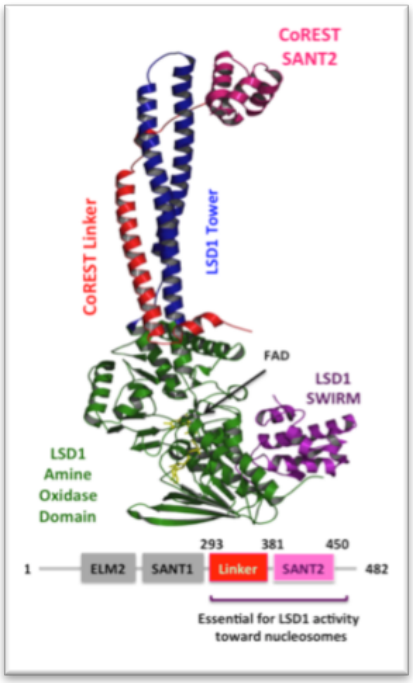
Introduction

Our goals for this project are to explore LSD1 inhibition of protein-protein interactions as a potential therapy for ERα$^-$ breast cancer, ameliorating iLCC for *in vivo* use, and using novel proteomics approaches to identify coregulatory proteins interacting with ERα and LSD1 in ERα$^+$ cells and deduce how the complement of LSD1 associating proteins change in ERα$^-$ cancers. We have made significant progress on the aims this project. There are no changes we wish to make to the Statement of Work.



Key Words

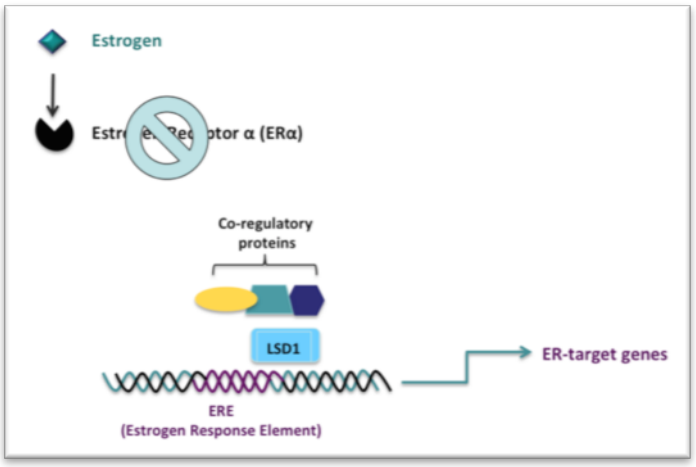
histone demethylase, estrogen receptor, CoREST, corepressor, estrogen-receptor positive breast cancer, estrogen receptor negative breast cancer, epigenetics, nuclear hormone receptor, estrogen

Overall Project Summary

The lysine specific histone demethylase 1 (LSD1, also known as KDM1A, see inset) is an important histone-modifying enzyme that regulates the expression of many genes important in breast cancer progression and proliferation. Present various transcriptional complexes paired with tightly-associated nucleosome targeting protein CoREST and the histone deacetylases HDACs 1 and 2, the LSD1/CoREST/HDAC complex is the core enzymatic machinery responsible for removing methyl marks on histone H3. The context to which the LSD1 complex acts is controlled by its interaction with partnering co-regulatory proteins that may direct access to specific methylation sites or influence LSD1 specificity.

By mechanisms that are far from well understood, estrogen binding to the estrogen receptor alpha (ER) undergoes conformational changes to facilitate co-regulatory protein association, then is recruited by components of the LSD1 multi-protein complex to translate nuclear hormone signaling into productive transcriptional activation of estrogen-responsive genes. In hormone-responsive breast cancers, ER activity is a proven target for pharmacological inhibition, as ER controls many cancer-associated proliferation genes.

However in highly aggressive breast cancers, ER production is abrogated, and thus by comparison there are limited therapeutic options for these aggressive cancers. In both breast cancers, LSD1 is highly expressed and activated and we have validated in cell culture that LSD1 is a potential target for both ER-positive and ER-negative cancers. In this proposal we wish to examine the molecular contributors that govern signal transmission between the ER complex and the LSD1/CoREST/HDAC1/2 complex to initiate transcriptional programs at ER target genes, to validate that LSD1 as a breast cancer target in both ER- and ER+ cancers target by examining the ability of our recently developed potent and selective inhibitor the LSD1-CoREST interaction to reduce tumor growth in a murine xenograft models of breast cancer. In year 1 of this proposal period, progress toward examining the efficacy of protein-protein interaction inhibitors of the LSD1-CoREST complex will be examined to determine the effect of loss of LSD1 function on ER signaling. In addition, our progress towards using mass spectrometry to probe the extend of the interaction of the ER with the LSD1 complex in order to understand the molecular basis for signal communication between hormone-liganded nuclear hormone receptors and epigenetic histone demethylase enzymatic machinery will be summarized. This work will pave the way for understanding more generally the interactions between nuclear hormone receptor complexes and epigenetic enzyme machinery that impacts chromatin remodeling and transcriptional regulation.



Our Specific Aims are stated below, and following each Aim a brief progress report indicating pertinent data and results is provided. Although there are no published papers in this initial year, several papers have been submitted for publication and as we finish our analyses of these data, we will be reporting our findings in the literature.

Progress on Aim 1:

Aim 1- We wish to unequivocally confirm that iLCC functions as an inhibitor of both ERα+ and ERα_ breast cancers *in vivo* in a clinically-relevant xenograft animal model of breast cancer.

In this aim, we proposed to analyze the effect of iLCC expression on LSD1 activity and on interruption of ER signaling and growth proliferation in ER-positive cells and on tumor growth arrest of ER-negative breast cancer cells. We have completed our proposed xenograft studies using the MCF7 ER+ cell line. We prepared lentavirus-infected MCF7 breast cancer cells that conditionally express iLCC fused to a Gal4 carrier under upon exposure tetracyclines such as doxocycline. The iLCC-Gal4 fusion remains effective at abrogating the CoREST-LSD1 interaction. We determined that iLCC was expressed in cell culture when exposed to doxycycline levels, and that expression of iLCC in MCF7 cells in cell culture resulted in decreased proliferation and increased histone H3K4 and H3K9 methylation levels, which is consistent with abrogating LSD1 activity. Expression of iLCC was confirmed by Western analyses. Expression of iLCC lead to proper nuclear localization of the peptide and pronounced stability, presumably due to protection from degradation following association to LSD1. Expression also inhibited the proliferation of MCF7 cells.

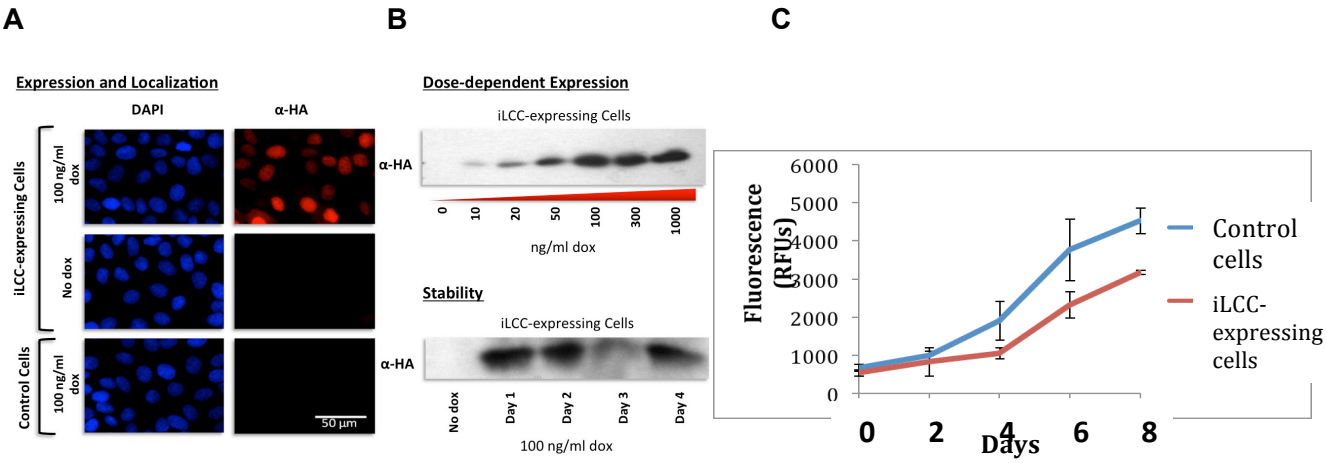
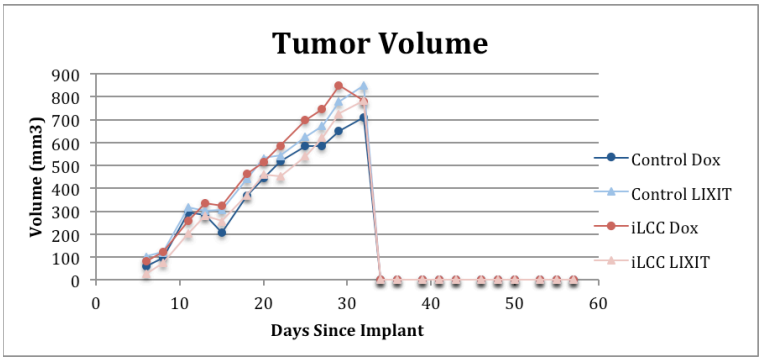


Figure 1. Doxycycline-induced expression of iLCC within MCF7 breast cancer cells. (A) Validation of nuclear localization of the expressed iLCC peptide; (B) Western blot of dose-dependent expression of iLCC and stability of iLCC; (C) Expression of iLCC leads to delays in MCF7 cell proliferation.

To examine the effects of iLCC expression in MCF7 cells within a xenograft implantation murine breast cancer model, doxycycline-inducible iLCC-expressing MCF7 cells were transplanted into nude mice that were exposed to estrogen and varous concentrations of dozycycline. As shown in Figure 2 below, a reduced proliferation rate was observed in vivo, yet there appeared to be a global increase in the methylation state of hstone H3K4 and H3K9. Interestingly, rather than shrink, tumors appeared to grow at normal or slightly below normal rates, but in many cases the morphology of the solid tumor dramatically changed to a satellite morphology, where smaller satellite tumors developed in dox-induced xenografts. The formation of such satellite tumors is consistent with early metastasis, which may suggest possible cross-inhibition of the MTA1/LSD1 complex. It is also possible that CoREST3 may play an antagonistic role as compared to CoRESTs 1 and 2, and all of these interact with LSD1 via the tower domain. Presently we are examining the effects of dox-inducible iLCC in ER_ breast cancer cell lines in order to complete the analysis of iLCC expression within ER+ and ER- breast cancer xenograft models. For the MCF7 study, 65 mice were used in this study broken-down into the following groups:

Control Cells + Dox: 16 mice
Control Cells - Dox: 15 mice
iLCC Cells + Dox: 17 mice
iLCC Cells - Dox: 17 mice

A



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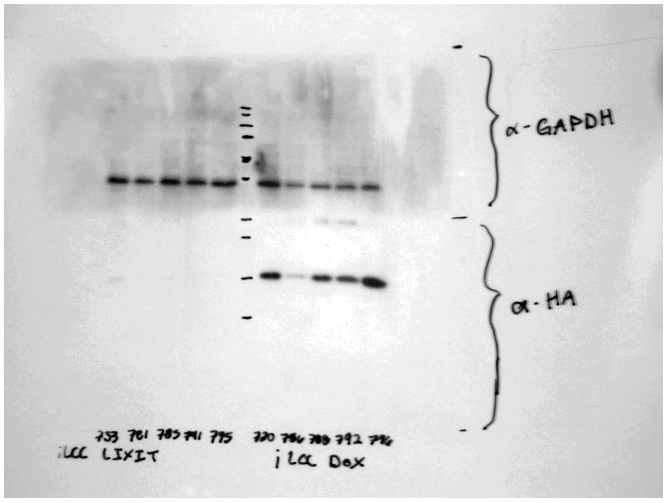


Figure 2. (A) Analysis of the effects of Dox-inducible iLCC expression on MCF7 tumor xenograft growth; (B) Western analysis indicating successful Dox-induced expression of iLCC in MCF7 implanted murine xenograft animal models of breast cancer.

We have conducted follow up analyses as are listed in the original proposal and statement of work for the analysis of tumors from mice with MCF7 xenograft implants and will compare these results to the MDA-MB-231 xenograft tumors knockdowns. Preliminary ChIP analyses from representative tumors are depicted in Figure 3 below.

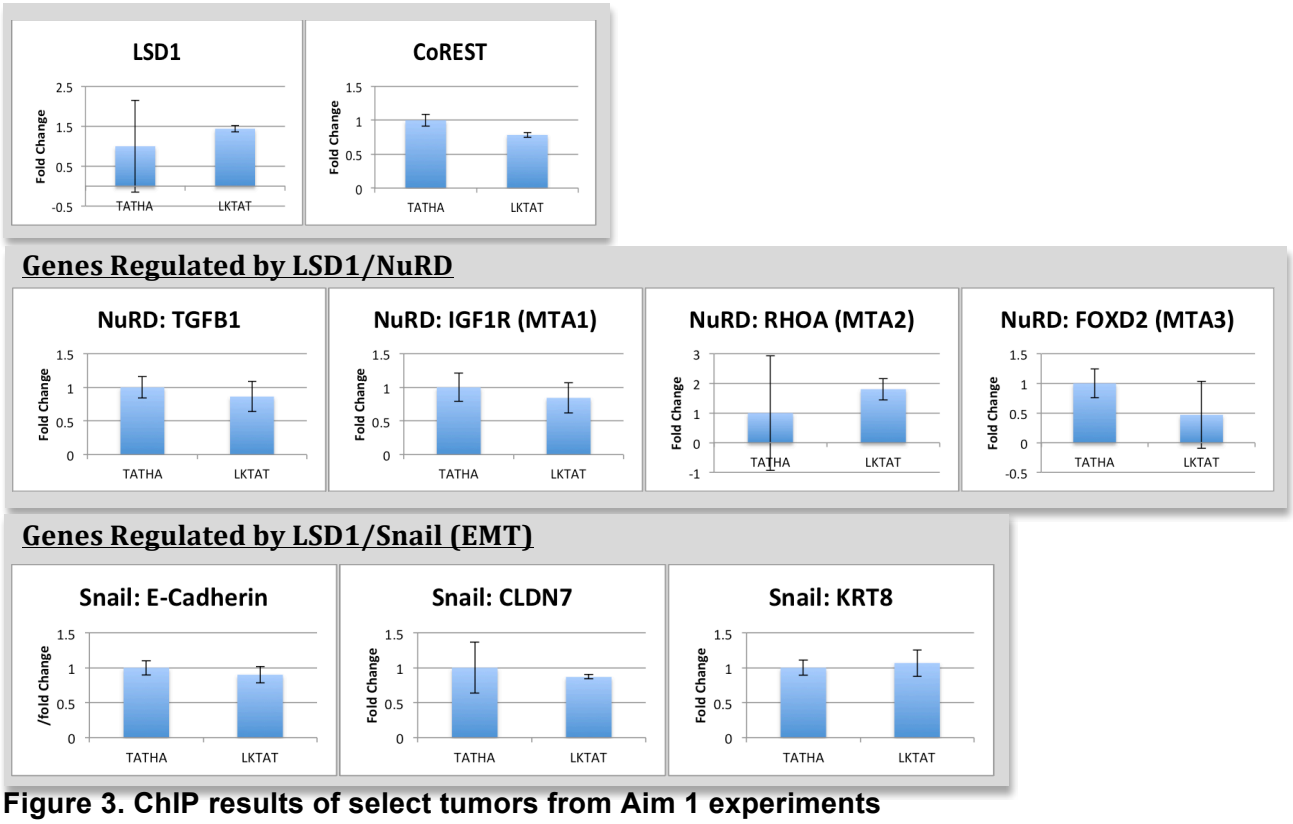


Figure 3. ChIP results of select tumors from Aim 1 experiments

As anticipated, histone methylation status at histone H3 at H3K4 and H3K9 increase with iLCC expression, which is consistent with LSD1 inhibition (Figure 4). We noticed in some primary tumor samples that high levels of doxycycline in controls increased histone methylation levels, and so are examining the expression level of histone methyltransferases, especially in cells treated with 1000 ng/mL DOX since elevated histone methyltransferase activity could mask inhibitory effects of LSD1 inhibition by iLCC. Similarly, we are examining

if the expression level of other demethylases with similar specificity patterns are down regulated during these studies.

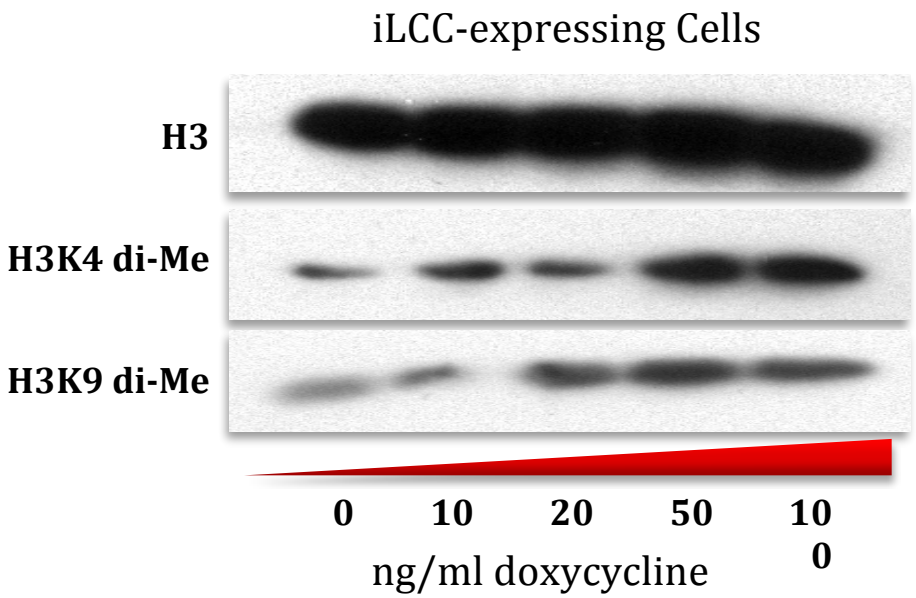


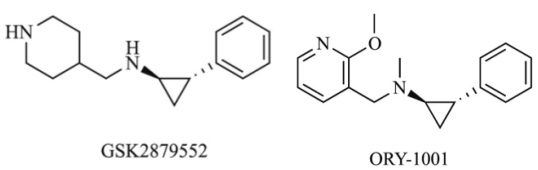
Figure 4. iLCC expression is associated with increased histone H3K4 and H3K9 methylated forms.

Progress on Aim 2.

Aim 2- Although iLCC is remarkably resistant to degradation *in cellulo*, we wish to also ameliorate its structure and develop formulations to that will reduce susceptibility to hydrolysis.

Although the apparent stability of iLCC is enhanced upon association with LSD1, with a half life in vivo of days, we have made progress in Aim 2 by synthesizing nonpeptidic small molecule LSD1 inhibitors that are selective for the LSD1 isoform. The structures of these recently disclosed selective LSD1 inhibitors are depicted below. In addition, to assist in the design of peptidomimetics, we have mapped the interface of CoREST and LSD1 using overlapping peptide library approaches, much like epitope mapping is to antibody ligand recognition analyses. In short it appears that short peptides have modest affinity for LSD1 along the CoREST linker helical interface, and we are now preparing stapled peptides to examine if the addition of these supportive crosslinks will stabilize the helical conformation sufficiently to enhance binding to LSD1.

A



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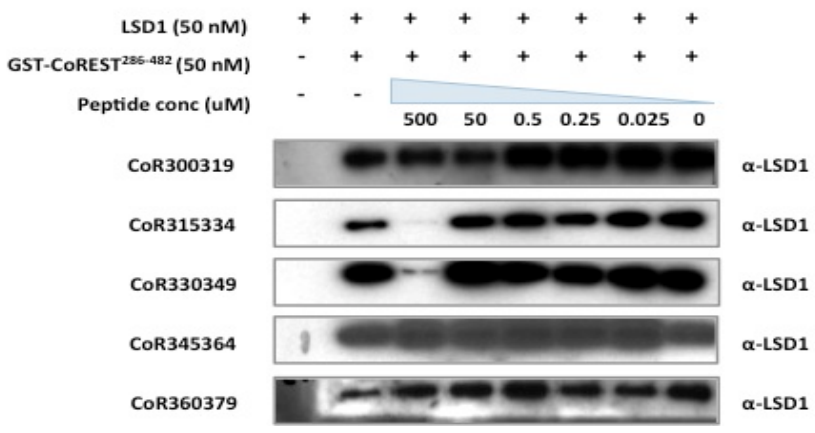

Figure 5. (A) Structure of LSD1-selective small molecule inhibitors; (B) Western blots of affinity pull down competition experiments to dissect the subdomain of CoREST for stapled peptide production.

Progress on Aim 3:

Aim 3- To assist in understanding LSD1 function in breast cancer, we wish to provide an innovative solution to identify ER targets involved in communication with LSD1 within complex protein mixtures by combining SPROX shotgun proteomics with chemical genetic probes of ER communication with the LSD1 complex (developed by the PI).

Mass spectrometry experiments to identify the physical basis for communication between the ER and LSD1 complex have been initiated. Unfortunately, because of the poor degree of fragmentation LSD1 exhibited using bottom up proteomics, insufficient coverage was achieved in initial MS analyses to facilitate SPROX studies with the ER. However, we have recently turned to using H/D exchange using high resolution ESI/MS methods which overcame the fragmentation issues. We have just completed preliminary analyses of LSD1 using binding to histone H3 as a perterbant, and am pleased to see that we have been able to easily visualize specific conformational changes within LSD1 upon substrate binding. Shown below in Figure 6 are structures of LSD1, before and after exposure to full length histone H3. Changes in color reflect increased exchange dymanics between enzyme and substrate. We are now pursuing similar analyses with LSD1 and CoREST fragments as well as newly produced recombinant full length CoREST. Once these data are in hand, we will be introducing the ER alpha or ER beta isoforms along with the LSD1-CoREST complex, and probing using H/D exchange to identify the binding interface, and will then carry out the remainder of the experiments outlined in Aim 3.

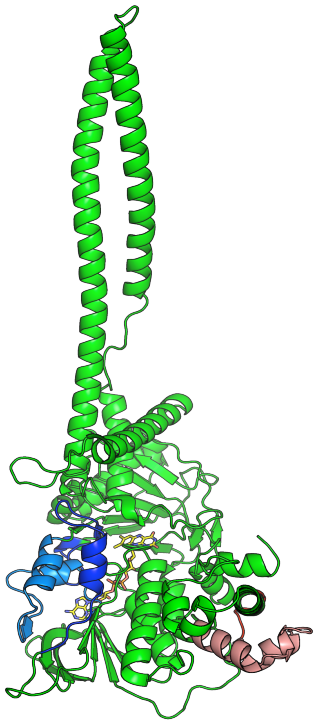

Figure 6. H/D exchange rates mapped onto the structure of LSD1 in the presence of a histone H3 binding partner. Blue illustrates areas with enhanced H/D exchange rates.

Key Research Accomplishments:

- Dox-induced expression of iLCC in MCF7 cells, and the subsequent characterization
- Dox-induced expression of iLCC within MCF7 cells in a murine xenograft model of breast cancer and their characterization
- Synthesis of isoform selective LSD1 inhibitors
- Successful fragmentation and sequencing of LSD1 by ESI FT-MS methods
- Use of H/D exchange mass spectrometry to identify functionally important interfaces between LSD1 and a partner protein

Conclusion
In short we have made substantial progress on the project and are excited to complete the experiments outlined in the statement of work for year 2 of this proposal period. Thank you for your generous support of this research project.

Publications, Abstracts and Presentations
Nothing to report yet for publications that are in press, but two articles are in the final stages of completion for a November submission

This work was presented as a research talk by Prof. McCafferty at the 2014 ASBMB conference on Transcription, in Snowbird, UT.

Inventions, Patents, and Licenses
Nothing to report

Reportable Outcomes
Nothing to report

Other Achievements
Nothing to report

References
Nothing to report

Appendices
Nothing to report